Research Article

High pressure homogenization of *Nannochloropsis oculata* for the extraction of intracellular components: Effect of process conditions and culture age

Carolina Shene[1,2], María Teresa Monsalve[1], Daniela Vergara[1], María Elena Lienqueo[3] and Mónica Rubilar[1]

[1] Department of Chemical Engineering, Center of Food Biotechnology and Bioseparations, BIOREN, Universidad de La Frontera, Temuco, Chile
[2] Centre for Biotechnology and Bioengineering (CeBiB), Universidad de La Frontera, Temuco, Chile
[3] Department of Chemical Engineering and Biotechnology, Centre for Biotechnology and Bioengineering (CeBiB), University of Chile, Santiago, Chile

Nannochloropsis is a genus of unicellular eukaryotes known primarily from the marine environment whose members are potential sources of lipids and long-chain polyunsaturated fatty acids; for the extraction of these and other valuable cell components, cell disruption is needed. High pressure homogenization (HPH) would be particularly suitable for microalgae with a recalcitrant cell wall such as *Nannochloropsis*. HPH conditions should be determined based on both the target cell component and properties of the cell suspension that in some cases are dependent on the age of the culture. The yields of soluble protein and total sugars from *N. oculata* ranged from 22.7 to 50.4 mg/g and from 55.0 to 62.5 mg/g, respectively, depending on HPH conditions (loading pressure and number of passes). The yield of the lipids extracted with the method of Bligh and Dyer was not affected by HPH conditions whereas lipids extracted with Soxhlet method ranged between 8.2 and 16.2%. Main fatty acids in the lipids extracted with the method of Bligh and Dyer and total lipids were palmitic acid (17.2 ± 0.1–23.0 ± 0.2%), palmitoleic acid (22.9 ± 0.3–19.1 ± 0.9%), and eicosapentaenoic acid (20.6 ± 0.3–29.2 ± 0.3%). HPH of *N. oculata* cells promoted a different effect on particle size distribution (PSD) depending on the age of the culture. HPH reduced cell aggregation observed in the 10 day cell suspension, whereas it promoted aggregation of the 30 day cell suspension.

Practical applications: The feasibility of producing a wide range of products from microalgae is determined by the culture conditions and the conditions of the stages in the downstream processing. Because main microalgae components are intracellular, a scalable cell disruption operation such as HPH is required. Cell disruption degree is determined not only by the equipment design and its operational conditions but also by the cell suspension properties. The results allowed us to conclude that a different combination loading pressure/number of passes in HPH maximizes the recovery of hydrosoluble compounds (proteins and sugars) and lipids in *N. oculata*. Besides, since the PSD of the microalgae suspension is a function of culture age, this variable could affect process productivity.

Keywords: Biodiesel / Cell disruption / Cell size / Lipid extraction / Microalgae / Protein solubility

Received: January 6, 2015 / Revised: April 15, 2015 / Accepted: May 19, 2015

DOI: 10.1002/ejlt.201500011

Correspondence: Carolina Shene, Department of Chemical Engineering, Center of Food Biotechnology and Bioseparations, BIOREN, Universidad de La Frontera, Casilla 54-D, Temuco, Chile
E-mail: carolina.shene@ufrontera.cl
Fax: +56 45 2732402

Abbreviations: ***a*,** parameter in the disruption kinetics; ***A*,** alkalinity; **ASW,** artificial seawater; ***C*,** cell concentration; ***F*,** mass flow rate; **FAME,** fatty acid methyl esters; **HPH,** high pressure homogenization; ***k*,** disruption rate constant; $L_{C/M}$, lipids extracted with chloroform/methanol; L_{PE}, lipids extracted with the Soxhlet method with petroleum ether; ***N*,** number of passes through HPH; **NFE,** nitrogen-free extract; ***P*,** electric power for HPH; **PSD,** particle size distribution; ΔP, pressure difference; ***R*,** fraction of soluble protein released after *N* passes through homogenizer; **RS,** reducing sugars; **SDS–PAGE,** sodium dodecyl sulfate polyacrylamide electrophoresis; **SP,** soluble protein; **TS,** total sugars; **vvm,** volume of air per volume of culture medium per minute; ***X*,** biomass concentration; η, overall efficiency of the pumping system; ρ, density of the microalgae suspension

1 Introduction

Microalgae are considered promising raw materials for different products; depending on the species, microalgae biomass contains lipids (triacylglycerols, polyunsaturated fatty acids, sterols), carbohydrates (glucose, starch, and other polysaccharides), pigments (chlorophyll, carotenoids, and phycobiliproteins), essential vitamins, and bioactive and health promoting compounds. Among the thousands of species that are believed to exist just a handful are cultivated in industrial quantities; for instance, species from genera *Chlorella*, *Dunaliella*, *Haematococcus*, *Arthrospira*, and *Aphanizomenon* [1].

Nannochloropsis is a genus of unicellular eukaryotes known primarily from the marine environment [2]. Species in this genus have been studied for the production of biodiesel due to the ease cultivation and the high lipid content (30–60% of dry biomass). *Nannochloropsis* species are relevant for the aquaculture; frozen and suspended concentrates of *N. oculata* are used in Japan for rotifer enrichment [3]. Compared with other microalgae used in aquaculture protein content and the content of essential amino acids in *Nannochloropsis* species are significantly higher [4]. Marine *Nannochloropsis* species could be an alternative source for the replacement of fish oil and the development of nutraceutical products because of the high content of omega-3 long-chain polyunsaturated fatty acids; the content of eicosapentaenoic acid (EPA, C20:5n-3) in the oils of *N. gaditana* and *N. oculata* has been reported to be 175 ± 12 and 193 ± 24 mg/g of oil, respectively [5]. Extraction of the valuable intracellular compounds accumulated by *Nannochloropsis* species is restricted due to the small cell size and the cell wall composition [6, 7]. Cell wall of *N. gaditana* consists of a cellulosic inner wall that is protected by an outer hydrophobic layer of algaenan (polyether-linked long-chain alkyl units) [8].

Among the non-chemical methods used for cell disruption are ultrasound [9], microwave, milling, and high pressure homogenization (HPH) [10]. Cell disruption by HPH is a method that would be particularly suitable for microalgae with recalcitrant cell wall. At industrial scale, HPH could be easily introduced after biomass harvest; moreover, this operation does not require chemical products. In a HPH unit, the cell suspension is forced to flow through a small orifice where mechanical effects, including turbulence, shear stress, and cavitation, promote cell lysis [11]. In addition to the working pressure and valve design, cell disruption in HPH is determined by the pressure at the valve (loading pressure) and the properties of cell suspension (viscosity, cell concentration, cell size) [12]. In the processing of other microorganisms using a multiple passes operation, the fraction of soluble protein released increases with the number of passes following a first order kinetics [13]; however, as the number of passes increases, costs also increase due to power consumption.

The objective of this work was to determine the effects of two operational conditions of a laboratory scale HPH unit on the subsequent recovery of hydrosoluble intracellular components (protein and sugars) and the extraction of lipids accumulated by *N. oculata*; the conditions were the loading pressure and the number of passes of the cell suspension through the HPH unit. Because lipids in microalgae are polar and apolar, the yield is dependent on the extraction method; to test this effect, the solid residue after HPH was extracted with the Soxhlet method using petroleum ether as a solvent and with the method of Bligh and Dyer [14]. The best combination of the operation conditions for HPH was used for the recovery of the studied components from *N. oculata* cells harvested at different times after inoculation (culture age). Particle size distribution (PSD) of the cell suspension before and after homogenization was determined to test the effect of culture age on cell disruption. Our results suggest that the conditions of HPH of *N. oculata* should be defined considering the component of interest; it was also found that the PSD of the homogenized cell suspension was dependent on the culture age.

2 Materials and methods

2.1 Microorganism and culture conditions

The strain *N. oculata* UTEX 2164 (University of Texas, Austin, USA) was used. Cultures were grown in f/2 medium modified (for 1 L: $NaNO_3$ 0.225 g, $NaH_2PO_4 \cdot 2H_2O$ 0.01695 g, trace mineral and vitamin solutions 300 μL each) in artificial sea water (ASW) [15]. Composition of the vitamin solution was for 1 L: cyanocobalamin (B12) 0.0005 g, thiamine HCl 0.1 g, biotin 0.0005 g. Composition of trace mineral solution was for 1 L: Na_2 EDTA 4.16 g, $FeCl_3 \cdot 6H_2O$ 3.15 g, $CuSO_4 \cdot 5H_2O$ 0.0100 g, $ZnSO_4 \cdot 7H_2O$ 0.022 g, $CoCl_2 \cdot 6H_2O$ 0.01 g, $MnCl_2 \cdot 4H_2O$ 0.18 g, $Na_2MoO_4 \cdot 2H_2O$ 0.006 g.

The biomass used to test the effect of HPH conditions was cultured outdoor in plastic bags (50 L) during 15 days (April–May, average irradiance 60 μmol photons m^{-2} s^{-1}). Biomass was harvested by centrifuging (6000×*g*, 10 min, 4°C) and the precipitates were frozen until all the mass needed for the HPH assays was produced. In the experiments conducted to test the effect of culture age, components of the modified f/2 medium were added to the sterile (121°C, 20 min) ASW. The inoculum (100 mL) was prepared in an Erlenmayer flask (250 mL) suspending 5 mL of a stock culture (kept at 4°C); incubation was performed for 10 days at 20 ± 2°C with artificial lighting (fluorescent tubes, 37.8 μmol m^{-2} s^{-1}) and light/dark cycles (12 h/12 h). The flask was manually stirred twice a day. The grown culture was transferred to a 3 L Erlenmayer flask containing 2 L of the sterile medium. Atmospheric air filtered under 0.2 μm was continuously bubbled at a rate of 0.6 vvm. Incubation conditions were the same as those described for the

inoculum. Samples were taken to determine cell concentration (cell count with hemocytometer), nitrate concentration (NitraVer® 5 nitrate reagent, Hach Company, Loveland, CO, USA) and alkalinity (acid titration to a pH of 4.2). Biomass was harvested after 10, 20, and 30 days from inoculation and immediately submitted to HPH.

2.2 Proximate composition

Proximate composition was determined according to the methods of the Association of Official Analytical Chemists [16]. Water was evaporated at 65°C. Ash content was determined following combustion at 550°C for 6 h. Lipids were assayed by petroleum ether extraction for 6 h (Soxhlet method). Crude fiber content was determined digesting the dry and de-fatted sample in acid (0.255 N H_2SO_4) and then in alkali (0.313 N NaOH). Protein content was determined by measuring nitrogen (Kjeldahl method); a nitrogen-to-protein conversion factor equal to 4.87 suggested for *N. oculata* [17] was used. Nitrogen-free extract was determined by difference. Lipid content after acid hydrolysis was determined using the Weibull–Stoldt method according to the AOAC official method of analysis 991.36 [16]; 10 mL of distilled water and 10 mL of HCl (35% w/v) were added to the dry sample (2 g). The mixture was heated to ebullition for 45 min after which 1 g of celite 545 (particle size 0.02–0.1 mm) (Merck, Darmstadt, Germany) was added. The hydrolyzed suspension was filtered through a celite bed (3 g in 20 mL of distilled water) deposited on a filter paper and washed to neutral pH. The filter with celite containing the adsorbed lipids was dried (105°C, 30 min) before Soxhlet extraction.

2.3 Homogenization

A laboratory HPH unit (Stansted Fluid Power Ltd., London, England) was used. Suspensions were prepared with the frozen biomass; the ratio of biomass and distilled water was 1.25 g:20 mL (dry solid content 1.6% w/w). The factors evaluated were the pressure exerted on the homogenizing valve (75/10,875, 125/18,125, and 230/33,350 MPa/psi) and the number of passes of the sample through the unit (1, 2, 4, and 6 passes). The homogenized sample was received in a beaker submerged in an ice bath to reduce the temperature before recirculation. The pH of the homogenized suspension was adjusted to 6 with 0.01 N HCl; the volume was brought to 50 mL with distilled water in a volumetric flask. The control was prepared following the same procedure using non homogenized biomass. The content of the solids in the suspension was gravimetrically determined after drying a known volume. The remaining solution was centrifuged (4000×*g*, 10 min, 4°C) and the supernatant was stored at −18°C until analysis of soluble components. The residue was dried for analyzing the lipid content. The disruption rate constant (k) for the soluble protein (SP) was computed fitting the data to a first-order equation [13]:

$$R = 1 - \exp(-k\Delta P^a N) \quad (1)$$

where R is the fraction of SP released after N passes through the homogenizer valve and a is a parameter whose value is dependent on the cell [13]. The electric power (P) required to pump the cell suspension through the valve was estimated from

$$P = \frac{F\Delta P}{\rho\eta} N \quad (2)$$

where F is the mass flow rate (kg/s), ρ is the density of the cell suspension (960 kg/m^3), and η is the overall efficiency of the pumping system. F was estimated from the volume of the HPH piston (10 mL) and the average time of discharge equal to 9 s.

2.4 Particle size distribution (PSD)

PSD of cells harvested at 10, 20, and 30 days were determined before and after HPH (125 MPa/6 passes) using a laser diffraction particle size analyzer (Sald – 3101, Shimadzu, Kyoto, Japan).

2.5 Analytical methods

Concentration of SP was measured using the methodology described by Lowry et al. [18] with bovine serum albumin as standard. The concentrations of total sugars (TS) and reducing sugars (RS) were determined using the phenol–sulfuric acid method [19] and dinitrosalicylic acid method [20], respectively. Yields of the hydrosoluble components were expressed as the mass per unit of dry solids in the suspension. The mass of Soxhlet extracted lipids with petroleum ether (L_{PE}) from the dry residue (1 g) was determined using 150 mL of the solvent. Chloroform/methanol was used to extract the lipids ($L_{C/M}$) according the Bligh–Dyer methodology [14]. After solvent evaporation, mass of the lipids was gravimetrically determined. Results were expressed as the mass of lipids per unit mass of the dry residue. To test the effect of pH on protein solubility, the defatted biomass sample (0.1 g) was added to a beaker containing 10 mL of distilled water whose pH was adjusted to different values (1–13) with 0.1 M HCl or 0.1 M NaOH. The suspension was incubated at 25°C under agitation for 24 h. SP concentration was measured as described before. Sodium dodecyl sulfate polyacrylamide electrophoresis (SDS–PAGE) of the SP from the biomass homogenized with 125 MPa/6 passes and 230 MPa/6 passes was performed according to the method of Laemmli [21]. The gel was fixed in trichloroacetic acid solution (12% w/v) for 1 h before staining with Coomassie Blue G-250 [22]. The stained gel was scanned using Image Scanner III (GE Healthcare).

Composition of the fatty acids in the lipids was determined by gas chromatography (GC-2010 Plus; Shimadzu, Kyoto, Japan). Lipids were directly transesterified with methanol/chloroform/HCl (10:1:1 v/v/v) to produce fatty acid methyl

esters (FAME) [23]. FAME in the hexane layer were collected by centrifuging at 4°C and applied to the gas chromatograph equipped with a flame ionization detector and a split injector, using a Rtx-2330 capillary column (60 m × 0.32 mm × 0.2 μm film thickness, Restek Corp., Bellefonte, PA, USA). Nitrogen was used as the carrier gas. The gas chromatography conditions were: oven temperature at 140°C for 5 min, increasing to 240°C at a rate of 4°C/min, holding for 15 min. The temperature of injector and detector was 260°C. FAME were identified with a 37-component FAME Mix (Supelco, Bellefonte, PA, USA) with methyl nonadecanoate (Sigma, St. Louis, MO, USA) as the internal standard.

2.6 Statistics

All the experiments were carried out in duplicate and the results are presented as the average ± standard deviation. The relative influence of the factors on the responses was determined from two and one way analysis of variance (ANOVA). The significance of the difference between averages was determined using Duncan's test at 5% confidence level.

3 Results

3.1 Effect of HPH conditions on the recovery of hydrosoluble and lipid fractions

Yield of SP significantly increased ($p < 0.05$) as the number of passes increased (Fig. 1a) for the three loading pressures tested. The highest yield of SP (50.4 ± 2.4 mg/g), obtained with 125 MPa/6 passes, was 231% higher than the yield from the unprocessed biomass (control) (15.2 ± 0.9 mg/g). The release of SP from *N. oculata* cells followed a first order kinetic (Eq. 1); values of parameter *a* were 3.0, 2.7, and 2.3 for loading pressures of 75, 125, and 230 MPa, respectively. The rate constant was estimated as 2.64×10^{-7} (MPa^{-a}). The SDS–PAGE of the SP released from the biomass homogenized with 230 MPa/6 passes did not show proteins with molecular weights higher than 40 kDa observed in the SP obtained with 125 MPa/6 passes (Fig. 2).

Only the loading pressure exerted a small but significant effect ($p < 0.05$) on yield of TS (Fig. 1b). On average, the yield of TS obtained with 75, 125, and 230 MPa was 58.2 ± 2.6, 61.5 ± 1.9, and 56.3 ± 2.1 mg/g, respectively. HPH conditions had significant effects ($p < 0.05$) on the yield of RS (Fig. 1c); from the biomass homogenized with 125 MPa/6 passes, the yield of RS (33.1 ± 0.8 mg/g) was 25% higher than the yield from the control biomass.

The yield of $L_{C/M}$ was not affected by HPH conditions (Fig. 3a) and the differences between the treatments and the control (29.1 ± 1.3%) were not significant ($p > 0.05$). Since for some applications, such as the production of biodiesel, neutral lipids are of interest the effect of HPH conditions on

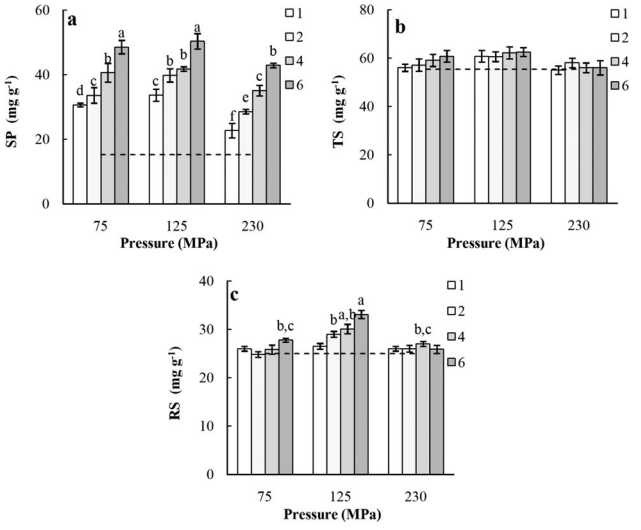

Figure 1. Effect of the conditions (loading pressure and passes) used for HPH of *N. oculata* on the yields of (a) soluble protein, SP, (b) total sugars, TS, and (c) reducing sugars, RS. Dotted line corresponds to the yield obtained from untreated biomass. Different letters denote that differences are significant ($p < 0.05$).

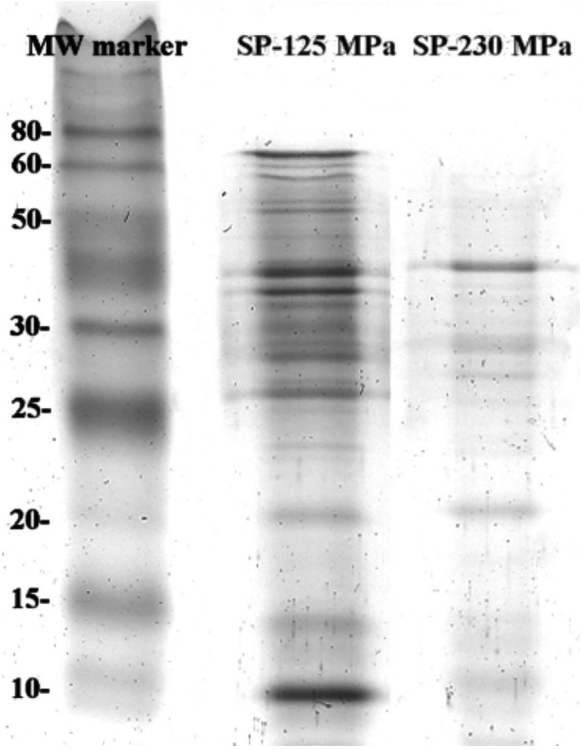

Figure 2. SDS–PAGE of the SP from the *N. oculata* homogenized with 125 MPa/6 passes (lane 2) and 230 MPa/6 passes (lane 3). Lane 1 shows the molecular weight marker.

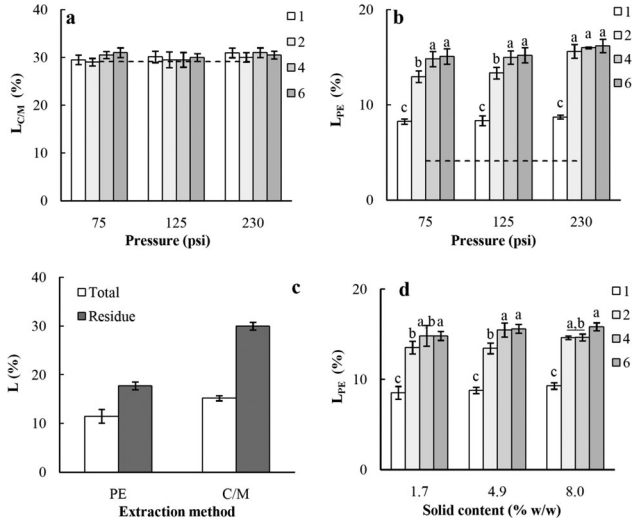

Figure 3. Effect of the conditions (loading pressure and passes) used for HPH of *N. oculata* on the yield of the lipids extracted with (a) chloroform/methanol (Bligh–Dyer methodology), $L_{C/M}$, and (b) Soxhlet with petroleum ether, L_{PE}. Graph (c) compares the yields of the lipids, extracted with the different methods, from the total homogenized biomass and the residue after homogenization (125 MPa/6 passes). Graph (d) shows the effects of the concentration of cell suspension and number of passes on the yield of L_{PE} from the total biomass homogenized at 125 MPa. Different letters denote differences are significant ($p < 0.05$).

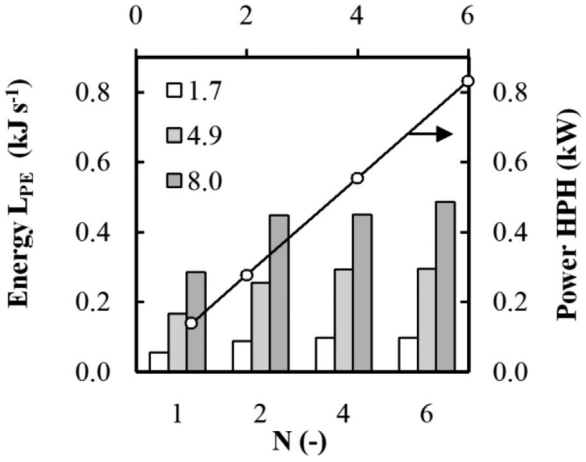

Figure 4. Effect of the number of passes (*N*) on the energy recovered from the Soxhlet extracted lipids for different concentrations of the cell suspension (data in Fig. 3d) and the power consumption for HPH with a loading pressure of 125 MPa.

the yield of Soxhlet extracted lipids (L_{PE}) was evaluated (Fig. 3b). HPH conditions had significant effects ($p < 0.05$) on the yield of L_{PE}. The highest yield of L_{PE} ($16.2 \pm 0.7\%$) was obtained from the residue of the suspension homogenized with 230 MPa/6 passes. The yield of L_{PE} from the control biomass was $4.1 \pm 1.4\%$ that increased to $12.1 \pm 0.7\%$ after the biomass was acid hydrolyzed. Compared with this latter value, HPH carried out at 230 MPa/6 passes allowed a 34% increase in the yield of L_{PE}. It is important to note that a direct comparison of the yield of lipids from the homogenized and the control is not possible. In the homogenization experiments, the soluble fraction of the biomass (SP, sugars, and salts) is transferred to the supernatant that is then separated by centrifuging; according to the results shown in the graphs in Fig. 1, this fraction ranged between 6.4 and 11.3% of the dry matter. The yields of the lipids obtained with both extractions methods, from the homogenized biomass (without eliminating the soluble fraction) and from the solid residue of the homogenized biomass (without the hydrosoluble components), were compared (Fig. 3c). The yield of the lipids, extracted with both methods, was significantly higher ($p < 0.05$) when this was measured in the residue. The effect of the concentration of cell suspension (1.7, 4.9, and 8.0% w/w) on the yield of L_{PE} in multiple passes HPH at 125 MPa was tested (Fig. 3d). Only the number of passes had a significant effect ($p < 0.05$) on the yield of L_{PE}. The results in Fig. 3d were used to calculate the energy that could be recovered in the lipids assuming a heating value of the crude algal lipids equal to 36 kJ/g [24] (Fig. 4). These values were compared with the energy consumed for HPH (Eq. 2). The ratio between the energy recovered in the lipids and that consumed for HPH was higher than 1 when one pass was used for the homogenization of the 4.9% w/w cell suspension or when the 8.0% w/w cell suspension was homogenized two times.

The comparison of the proximate composition of the biomass and the homogenized biomass (whole slurry after drying) is shown in Table 1. As expected, the contents of ash and crude fiber did not present significant differences ($p > 0.05$). The crude protein content in the untreated biomass ($29.2 \pm 0.1\%$) was similar to that in the

Table 1. Proximate composition of *N. oculata* biomass and homogenized biomass (125 MPa/6 passes) expressed on a dry matter basis.

Component (%)	Biomass	Homogenized biomass
Protein	29.7 ± 0.1	29.2 ± 0.1
Lipids	4.1 ± 1.4	7.8 ± 0.6
Fiber	1.3 ± 0.0	1.7 ± 0.1
Ash	19.1 ± 0.0	19.2 ± 0.1
NFE	45.8 ± 1.2	42.1 ± 0.6
Lipids[a]	12.1 ± 0.7	

NFE, nitrogen-free extract.
[a]After acid hydrolysis (Weibull–Stoldt method).

homogenized biomass (29.7 ± 0.1%). The content of SP in the microalgae biomass (22.7 ± 2.2–50.4 ± 2.4 mg/g) (Fig. 1a) was a small fraction (7.8–17.4%) of the total protein content. In part, this might be explained by the effect of pH on protein solubility. Protein solubility in a homogenized and defatted microalgae sample decreased (23%) as the pH of the suspension increased from 1 to 5, whereas as the pH increased from 9 to 13, the solubility increased more than 90%. Considering that the ratio between protein solubility at pH 13 and that at pH 6 (at which the hydrosoluble components were extracted from the homogenized biomass) was near 2, the yield of SP could reach a maximum value of 101 mg/g.

Following the AOAC methods, the content of lipids in the microalgae biomass was 4.1 ± 0.7%, while that in the homogenized biomass was 7.8 ± 0.6%. The same method applied to the acid hydrolyzed biomass yielded 12.1 ± 0.7%. Main fatty acids in the lipids extracted with the method of Bligh and Dyer and Soxhlet extracted from the acid hydrolyzed biomass were palmitic acid (17.2 ± 0.1–23.0 ± 0.2%), palmitoleic acid (22.9 ± 0.3–19.1 ± 0.9%), and EPA (20.6 ± 0.3–29.2 ± 0.3%) (Table 2).

Table 2. FAME in the lipids of *N. oculata* extracted with chloroform/methanol (Bligh–Dyer methodology), $L_{C/M}$, and Soxhlet extraction after acid hydrolysis ($L_{PE/AH}$)

Fatty acid		$L_{C/M}$	$L_{PE/AH}$
Butyric acid	C4:0	2.0 ± 0.9	1.0 ± 0.7
Caprylic acid	C8:0	0.4 ± 0.0	0.2 ± 0.0
Capric acid	C10:0	0.2 ± 0.0	0.1 ± 0.0
Lauric acid	C12:0	0.1 ± 0.0	0.2 ± 0.0
Tridecanoic acid	C13:0	ND	0.3 ± 0.0
Myristic acid	C14:0	2.7 ± 0.1	4.7 ± 0.1
Pentadecanoic acid	C15:0	0.2 ± 0.0	0.3 ± 0.0
Palmitic acid	C16:0	17.2 ± 0.1	23.0 ± 0.2
Palmitoleic acid	C16:1	22.9 ± 0.3	19.1 ± 0.9
Heptadecanoic acid	C17:0	0.3 ± 0.0	0.2 ± 0.0
Cis-10-heptadecenoic acid	C17:1n7	0.1 ± 0.0	ND
Stearic acid	C18:0	1.1 ± 0.1	0.4 ± 0.1
Oleic acid	C18:1n9c	4.5 ± 0.1	2.4 ± 0.1
Linolelaidic acid	C18:2n6t	ND	0.1 ± 0.1
Linoleic acid	C18:2	5.9 ± 0.1	2.9 ± 0.0
Arachidic acid	C20:0	0.3 ± 0.0	0.1 ± 0.0
γ-Linolenic acid	C18:3n6	0.3 ± 0.0	0.3 ± 0.1
Cis-11,14-eicosadienoic acid	C20:2	0.1 ± 0.0	0.2 ± 0.1
Behenic acid	C22:0	0.2 ± 0.0	0.6 ± 0.0
Cis-11,14,17-eicosatrienoic acid	C20:3n3	2.6 ± 0.1	2.6 ± 0.1
Cis-5,8,11,14,17-eicosapentanoic acid	C20:5n3	20.6 ± 0.3	29.2 ± 0.3
	Others	18.2 ± 0.3	12.3 ± 0.2

ND, not detected.

3.2 Effect of the culture age on the yield of cell components

N. oculata was cultivated during 30 days; cell growth, nitrate concentration, and alkalinity were followed (Fig. 5), and the yield of the different biomass components was determined at 10, 20, and 30 days (Table 3) after processing the cell suspension using 125 MPa/6 passes the condition that yielded the highest extraction of hydrosoluble components (Fig. 1). The yield of L_{PE} was not determined because of the small biomass concentration. The 10 day biomass yielded more SP and TS, whereas the highest yield of $L_{C/M}$ (34 ± 0.4%) was obtained from the 20 day biomass. The growth curve (Fig. 5) presented a 12 day growth period during which *N. oculata* grew at specific rate of 0.13 per day; nitrate consumption would explain the alkalinity increase during this period. The culture entered the stationary phase before nitrate exhaustion that occurred at day 24.

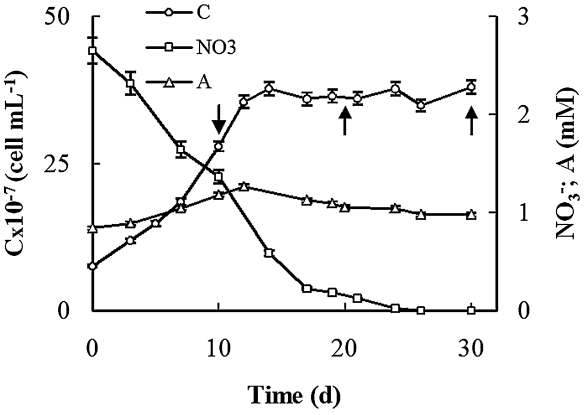

Figure 5. Changes of cell concentration (*C*), nitrate concentration (NO_3^-), and alkalinity (*A*) during the cultivation of *N. oculata* in modified f/2 medium in ASW. Arrows show the time at which cells were harvested for HPH.

Table 3. Biomass concentration (*X*) and yields of soluble protein, SP, total sugars, TS, and lipids extracted with chloroform/methanol (Bligh–Dyer methodology), $L_{C/M}$, from *N. oculata* biomass harvested at different times from inoculation (culture age) subjected to homogenization. SP and TS were quantified in the supernatant of the homogenized suspension and $L_{C/M}$ in the residue after drying. Homogenization conditions were 125 MPa/6 passes

Culture age (days)	X (g/L)	SP (mg/g)	TS (mg/g)	$L_{C/M}$ (%)
10	0.16 ± 0.01	29.0 ± 1.6[a]	43.1 ± 1.7[a]	30.9 ± 2.0[b]
20	0.18 ± 0.01	27.1 ± 1.1[a]	42.8 ± 1.7[a]	34.0 ± 0.4[a]
30	0.18 ± 0.02	21.7 ± 1.7[b]	34.4 ± 0.9[b]	33.6 ± 0.6[a]

In a column, different letters denote differences are significant ($p < 0.05$),

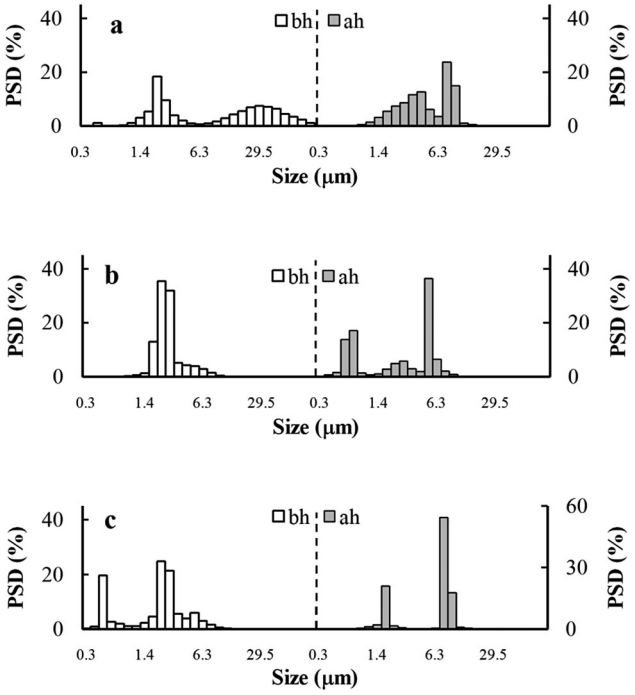

Figure 6. Particle size distribution (PSD) of *N. oculata* suspension before (bh) and after homogenization (ah) (125 MPa/6 passes). Cell suspension harvested after (a) 10 days, (b) 20 days, and (c) 30 days.

To test the effect of HPH on cell suspensions of different ages, the PSD of the suspensions was determined before and after HPH (Fig. 6). The results showed that the PSD of the cell suspension changed with culture age and that HPH promoted a different effect on PSD depending on the age of the culture. The PSD of the 10 day cell suspension was bimodal with peaks at 2.1 and 29.5 μm. Light microscopic observation showed cell aggregation that explained the fractions of high sizes. HPH reduced aggregation decreasing the average particle size to 3.3 ± 0.1 μm (Fig. 6a). Cell aggregation was not observed in the 20 day suspension (Fig. 6b); however, HPH promoted cell/debris aggregation. This phenomenon was more important in the homogenized 30 d cell suspension where the average particle size increased from 1.5 ± 0.1 to 3.3 ± 1.2 μm (Fig. 6c).

4 Discussion

N. oculata cells were homogenized in a laboratory scale HPH at loading pressures in the range of that utilized for cell disruption (55–200 MPa) [25]. In multiple passes HPH, a first-order kinetics is used to model the fraction of SP released; in this model, the SP fraction and the N are related through the loading pressure raised to an exponent whose value would be a measured of cell resistance to disruption [13]. For the *N. oculata* cells, parameter a decreases as the loading pressure increases an effect that can be ascribed to a reduction of protein solubility due to the increase of the suspension temperature in multiple passes HPH. Besides the reduction of SP yield at the highest loading pressure, the molecular weight of SP in the homogenate also presents differences. The release of SP after HPH represents a fraction (35%) of the total protein content in the biomass, a result that could be explained by the different methods used to measure SP (colorimetric method) and total protein (nitrogen by Kjeldahl). Kjeldahl's method uses a factor to convert the total nitrogen content into total protein. For microalgae, this factor strongly depends on the specie and growth conditions and ranges between 4.78 [17] and 5.95 [26].

The fact that the yields of SP and RS present similar patterns as the loading pressure increases could suggest the development of some type of interactions that decrease their solubility. The ratio between RS and TS, found between 0.43 and 0.53, indicates the presence of hydrosoluble polysaccharides. Polysaccharides described in *Nannochloropsis* strains are cellulose, component of the cell wall, and chrysolaminarin a water-soluble polysaccharide [27]. In spite of SP and sugars should not be found in the liquid phase of the non-homogenized biomass because these are mainly retained inside the cells, these were detected (data of control experiments in Fig. 1). These results could be ascribed to the experimental conditions that promoted some degree of cell damage due to freeze/thawing and the reduction of the ionic strength (biomass cultivated in ASW). Nevertheless, when intact cells were submitted to freeze/thawing cycles, the yield of SP did not show an increase (not shown).

HPH has no effect on the yield of the lipids extracted with chloroform/methanol (Bligh-Dyer methodology). This solvent mixture is commonly used to extract total lipids; chloroform extracts mainly neutral lipids (triglycerides, diglycerides, and waxes), while methanol extracts cell-membrane lipids (phospholipids, glycoproteins, and cholesterol). The extraction of cell-membrane lipids by methanol can increase cell permeability enhancing the extraction of intracellular lipids, an effect that would explain the absence of HPH effect on the yield of $L_{C/M}$. On the contrary, HPH increases the yield of Soxhlet extracted lipids. Complex matrices such as food and cereals typically require pretreatments such as acid hydrolysis prior to lipid extraction by Soxhlet method. Nevertheless, for *N. oculata* cells, the yield of L_{PE} after two or more passes of HPH is higher than the yield from acid hydrolyzed biomass. The extraction method determines the FAME composition of the lipids; the L_{PE} from the acid hydrolyzed biomass contain a higher proportion of palmitic acid and EPA compared with that in $L_{C/M}$ extracted from the control biomass. The lipid extraction methods evaluated have a disadvantage that might exclude their use in a large scale process; both require dry biomass. Drying of the harvested microalgae biomass with

moisture content higher than 80% could only be considered for high value products. In the production of low value products, such as biodiesel, the use of HPH for cell disruption will depend on the power consumption and the energy in the recovered lipids [28]. While the power consumption depends on the loading pressure and N, the energy associated to the lipids is mainly function of the lipid content in the biomass and the concentration of cell suspension. Only concentrated cell suspensions can justify the choice of high loading pressures and/or multiple passes for HPH. The results showed that a concentration of cell suspension equal or lower than 8.0% w/w does not affect the yield of L_{PE}. Thus, for the relatively low lipid content (less than 20% w/w) in the microalgae biomass, the concentration of cell suspension should at least 4.9% w/w when a single pass HPH at a loading pressure of 125 MPa is used. However, with these conditions, only 55% of the L_{PE} are recovered. To increase the lipid recovery, HPH should be carried out in multiple passes; if two passes are used at the same loading pressure, more than 92% of the L_{PE} can be recovered. However, the energy in lipids recovered is higher than the energy consumed for HPH when the concentration of the cell suspension is in this case 8.0% w/w.

The yield of the different cell components in *N. oculata* is determined not only by the conditions of HPH but also by the age of the culture. For the culture conditions used, the 20 day biomass presents the highest yield of $L_{C/M}$ ($34 \pm 0.4\%$). *N. oceanica* IMET-1 cultivated during 23 days with continuous illumination and CO_2-enriched air (1.5–2.0% v/v) is able to produce 69% lipid (dry weight) [29]. The lower lipid content in our experiments would be explained by the use energy reserves, mainly lipids, during the dark period (light/dark cycles of 12 h/12 h). Accumulation of neutral lipids in microalgae would occur after nutrient exhaustion (nitrogen and phosphorous), an stress condition that reduces cell multiplication [30, 31]. However, in *N. gaditana* and *N. oceanica*, nitrogen starvation down-regulates genes involved in carbon fixation and photosynthesis [27, 31]. Photosynthetic metabolism can also be modulated by the self-shading that increases as the cell concentration increases. Limitation in the supply of radiant energy constrains the rate of transport of inorganic carbon and reduces cell capacity to fix CO_2 [32]. In our case, the yield of $L_{C/M}$ increases during the active growth period when the culture was not limited by nitrate.

The yield of the different microalgae components is dependent on the biomass composition that changes as the age of the culture increases. Cell resistance to HPH can also change during the cultivation period because it has been reported that in some algae the thick of the cell wall increases as the cell becomes older [33]. The response of a cell suspension to HPH can be deduced from the changes in PSD; the size distribution of a homogenized cell suspension can have a smaller average size due to cell disruption, a larger average size due to aggregation [7], or be more complex presenting for instance a bimodal distribution with peaks representing the debris and cells. On the other hand, PSD of a cell suspension can be determined by the cell metabolism in response to culture conditions and medium composition that is a function of culture age. The increase of cell volume of *N. gaditana* in aged cultures has been reported [34], whereas sustained oscillations in the average cell size were found in continuous photoautotrophic cultures of *Chlorella vulgaris* an effect attributed to the synthesis of an inhibitor of the second round of initiation of DNA replication [35]. Another example is cell aggregation due to polysaccharide exudation triggered by high light intensity and high diurnal temperatures in summer outdoor cultures of *Nannochloropsis* sp. [36]. The results showed that *N. oculata* suspensions of different ages have different PSD and the effect of HPH on PSD is also different. Under the assumption that the yield of the different microalgae components is dependent on the PSD of the homogenized cell suspension, the HPH conditions should be adjusted taking into account the age of the culture.

5 Conclusions

The yield of hydrosoluble components (SP, TS, and RS) accumulated in *N. oculata* cells is dependent on the conditions of HPH, while the effect of homogenization conditions on the yield of lipids is dependent on the extraction method. Multiple passes HPH of *N. oculata* cells increased the yield of the Soxhlet extracted lipids, an effect that is not dependent on the concentration of the cell suspension. The different PSD of homogenized cells harvested at different ages might suggest that HPH conditions have to be chosen considering this characteristic of the biomass.

We are grateful for financial support from Conicyt through Fondecyt Project N° 1130500, Dirección de Investigación at Universidad de La Frontera for the economic support provided through Convenios de Desempeño I, II, and GAP. The authors also acknowledge the financial support given by Consortium Desert Bioenergy S.A. for research and development of biofuels from microalgae, INNOVA Chile-CORFO 09CTEI-6860, subprogram 5 "Optimization processes for the extraction and recovery of lipids from microalgae biomass."

References

[1] Spolaore, P., Joannis-Cassan, C., Duran, E., Isambert, A., Commercial applications of microalgae. *J. Biosci. Bioeng.* 2006, *101*, 201–211.

[2] Fawley, K. P., Fawley, M. W., Observations on the diversity and ecology of freshwater *Nannochloropsis* (Eustigmatophyceae), with descriptions of new taxa. *Protist* 2007, *158*, 325–336.

[3] Guedes, A. C., Malcata, F. X., Nutritional value and uses of microalgae in aquaculture. *Aquaculture* 2012, *1516*, 59–78.

[4] Welladsen, H., Kent, M., Mangott, A., Li, Y., Shelf-life assessment of microalgae concentrates: Effect of cold preservation on microalgal nutrition profiles. *Aquaculture* 2014, *430*, 241–247.

[5] Ryckebosch, E., Bruneel, C., Termote-Verhalle, R., Goiris, K., et al., Nutritional evaluation of microalgae oils rich in omega-3 long chain polyunsaturated fatty acids as an alternative for fish oil. *Food Chem.* 2014, *160*, 393–400.

[6] Olmstead, I. L. D., Kentish, S. E., Scales, P. J., Martin, G. J. O., Low solvent, low temperature method for extracting biodiesel lipids from concentrated microalgal biomass. *Bioresource Technol.* 2013, *148*, 615–619.

[7] Spiden, E. M., Yap, B. H. J., Hill, D. R. A., Kentish, S. E., et al., Quantitative evaluation of the ease of rupture of industrially promising microalgae by high pressure homogenization. *Bioresour. Technol.* 2013, *140*, 165–171.

[8] Scholz, M. J., Weiss, T. L., Jinkerson, R. E., Jing, J., et al., Ultrastructure and composition of the *Nannochloropsis gaditana* cell wall. *Eukaryot. Cell* 2014, *13*, 1450–1464.

[9] Gerde, J. A., Montalbo-Lomboy, M., Yao, L., Grewell, D., Wanga, T., Evaluation of microalgae cell disruption by ultrasonic treatment. *Bioresour. Technol.* 2012, *125*, 175–181.

[10] Halim, R., Rupasinghe, T. W. T., Tull, D. L., Webley, P. A., Mechanical cell disruption for lipid extraction from microalgal biomass. *Bioresour. Technol.* 2013, *140*, 53–63.

[11] Middelberg, A. P. J., in: Desai M. A. (Ed.), *Downstream Processing of Proteins: Methods and Protocols*, Vol. 9, Humana Press Inc, Totowa, NJ 2000.

[12] Lee, A. K., Lewis, D. M., Ashman, P. J., Disruption of microalgal cells for the extraction of lipids for biofuels: Processes and specific energy requirements. *Biomass Bioenerg.* 2012, *46*, 89–101.

[13] Follows, M., Hetherington, P. J., Dunnill, P., Lilly, M. D., Release of enzymes from bakers' yeast by disruption in an industrial homogenizer. *Biotechnol. Bioeng.* 1971, *13*, 549–560.

[14] Bligh, E. G., Dyer, W. J., A rapid method for total lipid extraction and purification. *Can. J. Biochem. Physiol.* 1959, *37*, 911–917.

[15] Shene, C., Leyton, A., Rubilar, M., Pinelo, M., et al., Production of lipids and docosahexaenoic acid by a native *Thraustochytrium* strain. *Eur. J. Lipid Sci. Technol.* 2013, *115*, 890–900.

[16] AOAC. *Official Methods of Analysis of Official Analytical Chemists International*, 16th Ed., Assoc. of Official Analytical Chemists, Arlington, VA 1995.

[17] Lourenço, S. O., Barbarino, E., Lanfer Marquez, U. M., Aidar, E., Distribution of intracellular nitrogen in marine microalgae: Basis for the calculation of specific nitrogen-to-protein conversion factors. *J. Phycol.* 1998, *34*, 798–811.

[18] Lowry, O. H., Rosebrough, N. J., Fan, A. L., Randall, R. J., Protein measurement with Folin phenol reagent. *J. Biol. Chem.* 1951, *193*, 256–275.

[19] Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., Smith, F., Colorimetric method for determination of sugars and related substances. *Anal. Chem.* 1956, *28*, 350–356.

[20] Miller, G. L.Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* 1959, *31*, 426–428.

[21] Laemmli, U. K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970, *227*, 680–685.

[22] Neuhoff, V., Arold, N., Taube, D., Ehrhardt, W., Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis* 1988, *9*, 255–262.

[23] Lewis, T. E., Nichols, P. D., McMeekin, T. A., Evaluation of extraction methods for recovery of fatty acids from lipid-producing microheterotrophs. *J. Microbiol. Methods* 2000, *43*, 107–116.

[24] Sharma, P., Khetmalas, M.B., Tandon, G.D., in: Salar, K. D., Gahlawat, S. K., Siwach, P., Duhan, J. S. (Eds.), *Biotechnology: Prospect and Applications*, Springer, India, 2013.

[25] Geciova, J., Bury, D., Jelen, P., Methods for disruption of microbial cells for potential use in the dairy industry—a review. *Int. Dairy J.* 2002, *12*, 541–553.

[26] González López, C. V., Cerón García, M. C., Acién Fernández, F. G., Segovia Bustos, C., et al., Protein measurements of microalgal and cyanobacterial biomass. *Bioresour. Technol.* 2010, *101*, 7587–7591.

[27] Dong, H. P., Williams, E., Wang, D. Z., Xie, Z. X., et al., Responses of *Nannochloropsis oceanica* IMET1 to long-term nitrogen starvation and recovery. *Plant Physiol.* 2013, *162*, 1110–1126.

[28] Yap, B. H. J., Dumsday, G. J., Scales, P. J., Martin, G. J. O., Energy evaluation of algal cell disruption by high pressure homogenization. *Bioresource Technol.* 2015, *184*, 280–285.

[29] Xiao, Y., Zhang, J., Cui, J., Feng, Y., Cui, Q., Metabolic profiles of *Nannochloropsis oceanica* IMET1 under nitrogen-deficiency stress. *Bioresour. Technol.* 2012, *130*, 731–738.

[30] Vieler, A., Wu, G., Tsai, C-H., Bullard, B., et al., Genome, functional gene annotation, and nuclear transformation of the heterokont oleaginous alga *Nannochloropsis oceanica* CC MP1779. *PLoS Genet.* 2012, *8*, e1003064.

[31] Radakovits, R., Jinkerson, R. E., Fuerstenberg, S. I., Tae, H., et al., Draft genome sequence and genetic transformation of the oleaginous alga 807 *Nannochloropis gaditana*. *Nat. Commun.* 2012, *3*, 686–696.

[32] Beardall, J., Johnston, A., Raven, J., Environmental regulation of CO_2-concentrating mechanisms in microalgae. *Can. J. Bot.* 1998, *76*, 1010–1017.

[33] Smith, D., Muscatine, L., Lewis, D., XI. Addendum. Biological Rev. 1969, 44, 86–90.

[34] Lubián, L. M., Montero, O., Moreno-Garrido, I., Huertas, I. E., et al., *Nannochloropsis* (Eustigmatophyceae) as source of commercially valuable pigments. *J. Appl. Phycol.* 2000, *12*, 249–255.

[35] Javanmardian, M., Palsson, B. O., Continuous photoautotrophic cultures of the eukaryotic alga *Chlorella vulgaris* can exhibit stable oscillatory dynamics. *Biotechnol. Bioeng.* 1992, *39*, 487–497.

[36] Chini Zittelli, G., Lavista, F., Bastianini, A., Rodolfi, V., Vincenzini, M. M. R., Tredici production of eicosapentaenoic acid by *Nannochloropsis* sp. cultures in outdoor tubular photobioreactors. *J. Biotechnol.* 1999, *70*, 299–312.

